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Therapeutic Effects of Ribavirin Given by the Intraperitoneal or Aerosol Route Against Influenza Virus Infections in Mice

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Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is an effective antiviral agent against type A influenza infection of mice. Therapy was most effective when administered as a small-particle aerosol early in the infection. Treatment was also effective by either the intraperitoneal or aerosol route in mice with histological evidence of pneumonia. Ribavirin increased the percent survival, lowered lung virus titers, and decreased the development of lung pathology when therapy was initiated at 6 h as a small-particle aerosol. There was no evidence of pulmonary toxicity or immunosuppressive effects.

Virus diseases of man and animals represent an important group of disease entities. Notwithstanding the influenza pandemic of 1918 to 1919, which was accompanied by great loss of life, influenza, like most virus diseases, is usually self-limiting and not associated with high mortality unless complicated by bacterial pneumonia. In 1957, Horsfall estimated that man suffers with viral diseases for 7 years of a 70-year life span (5). Loosli has stated that influenza is the major incapacitating viral disease not adequately controlled by vaccines (9).

Ribavirin has antiviral activity against both ribonucleic and deoxyribonucleic acid viruses (6, 13, 17). The demonstration that small-particle aerosols containing rimantadine or amantadine-hydrochloride were effective for the treatment of influenza virus-infected mice (15, 16) and the reported efficacy of parenterally injected ribavirin against lethal influenza infections in mice (1, 7) suggested that aerosols of ribavirin might be useful for the treatment of influenza. This report describes the therapeutic efficacy of ribavirin given in small-particle aerosols to treat experimental influenza virus infection in mice.

MATERIALS AND METHODS

Mice. Five-week-old outbred, female mice, Tac:(SW)fBR, were used for all experiments. Upon arrival, mice were housed 15 to a cage in random order. Each group of mice contained a separate subgroup destined for the same treatment, but reserved for serial sacrifice studies in addition to those recorded for survival. Groups contained 55 mice except that continuously treated groups contained 40 mice.

Virus. The mouse-adapted variant of the A/Aichi/2/68 (H3N2) strain of influenza virus used to infect the mice has been previously described (12, 15).

Lung virus titers. Lung samples were homogenized in 4.5 ml of heart infusion broth and assayed in 10- to 12-day-old embryonated eggs (15). Lung titers, expressed as the mean egg infective dose per lung, are the geometric mean of three individual mouse lungs at the indicated times postexposure. When no virus was detected in a lung sample, a value of 1.0 was assigned to calculate the geometric mean titer.

Aerosol sampling and dissemination system. The dissemination system used to infect the mice and for intermittent therapy has been previously described (15). The aerosols for continuous therapy were generated by a modified Collison system developed in our laboratory. The particles generated by this system have a mass median diameter of 1.4 μm, with 95% less than 5.2 μm (unpublished data, our laboratory). The relative humidity in the exposure chamber prior to treatment was 56.8 ± 1.9% standard error of the mean. After exposure, the relative humidity increased to 65.3 ± 1.7% for the intermittent method and approximately 70.0% for the continuous method.

Aerosol samples were collected for virus assay and quantitative analysis of ribavirin (15). The concentration of ribavirin per milliliter of collecting fluid in the glass impingers was determined by ultraviolet spectroscopy at a wavelength of 205 nm with a Beckman model DBG spectrophotometer. A standard curve was prepared by measuring the absorbance of known quantities of ribavirin.

Drug and treatment schedules. Ribavirin, obtained from the Nucleic Acid Research Institute of ICN Pharmaceuticals, Inc., Irvine, Calif., was dissolved in sterile triple-distilled water before aerosol or intraperitoneal (i.p.) administration. Injections (i.p.) were given once each day at a dose of 16 mg/kg per day. Aerosol treatments were either intermittent (80 min/day) or continuous (22 h/day). The dosage of the two aerosol methods of treatment was adjusted by using a spray suspension containing either 100 or 20 mg of ribavirin per ml for the intermittent and continuous methods, respectively. The

retained aerosol dose (50% of presented dose) for intermittent aerosol (80 min/day) was 21.5 mg/kg per day, whereas that for the continuously disseminated aerosol was 58 mg/kg per day, based on the calculated inhaled dose (14) and the retention properties of small-particle aerosols (4). Virus control mice were sham treated with sterile triple-distilled water for 80 min/day.

Two treatment schedules were followed. In one, therapy was initiated at 6 h postexposure to the virus and continued through 4 days. The second schedule involved initiation of therapy 3 days postexposure to the virus and continued through 7 days.

Gross lung lesion scores and histopathology. Five mice were killed on days 1, 2, 4, 6, 7, and 9 postinfection; the lungs were examined for gross lesions. The degree of pathological change was scored by the method of Fazekas de St. Groth and Donnelley (2) with the modification that dead animals were not included. Complete lung consolidation was scored as a 4 under this system. The values plotted represent the mean lung lesion scores of five mice from each group for each day.

Three mice were killed from each group 7, 14, and 21 days postexposure for histological examination. The lungs were fixed and processed as previously described (15).

Data. The percentage of survival was based on deaths occurring on days 5 through 21 after aerosol exposure to the virus. Mice used for survival data were separate from those used for virus titers or pathology examination. Intergroup comparisons were made using chi-square analysis for survival and one-way analysis of variance and Fisher's protected least significant difference test (13) for time to death. The mean time of death was calculated using only mice dying during the observation period. Survivors were not included in the calculations. Survival and time-to-death data from two replicate experiments were not statistically different and were combined.

RESULTS

Survival and MTD. Significant increases in percent survival ($P < 0.005$) occurred in all groups of mice given ribavirin regardless of the method of treatment or time of initial treatment (Table 1). There was no difference between the percentage of the survival of mice treated with continuous or intermittent small-particle aerosols when treatment was initiated at 6 h. Both aerosol methods of treatment resulted in significantly greater survival ($P < 0.005$) than the i.p. method of treatment. When treatment was initiated at 3 days, survival following all methods of therapy was significantly greater than that of the sham-treated virus control mice ($P < 0.005$). The aerosol methods were not superior to i.p. treatment when therapy was initiated on day 3. Treatment initiated at 6 h resulted in significantly greater percent survival ($P < 0.005$, by analysis of variance) than the same method of treatment initiated at 3 days.

Treatment (i.p.) initiated at 6 h did not significantly affect the mean time-to-death (Table 1). However, the time-to-death of mice treated from days 3 through 7 was significantly greater ($P < 0.01$) than for sham-treated virus control mice regardless of the method of treatment.

Virus titers. When therapy was initiated at 6 h, the peak lung virus titer of mice treated with continuously disseminated aerosols was significantly lower ($P < 0.05$) than the peak virus titer of sham-treated virus control mice at 48 h (Fig. 1, top). At 96 and 144 h, both intermittent and continuous aerosol methods of treatment resulted in significantly lowered lung virus ti-

TABLE 1. *Effect of ribavirin on survival and time-to-death of mice challenged with influenza virus by the aerosol route*[a]

Therapy route	Regimen	Survival		Mean time to death ± SEM[b] (days)
		Survivors/total	%	
Treated 6 h to 4 days				
Sham treated	80 min/day	27/109	24	7.6 ± 0.2
i.p.	1/day	62/110	56[c]	8.3 ± 0.3
Aerosol	80 min/day	105/110	95[c,d]	11.0 ± 1.4[e]
Aerosol	22 h/day	76/79	96[c,d]	11.3 ± 1.5[e]
Treated 3 to 7 days				
Sham treated	80 min/day	19/110	17	7.2 ± 0.1
i.p.	1/day	46/110	41[c]	8.8 ± 0.3[c]
Aerosol	80 min/day	59/110	53[c]	9.6 ± 0.5[c]
Aerosol	22 h/day	38/79	48[c]	9.4 ± 0.6[c]

[a] Challenge dose (mean egg infective dose per mouse) was $10^{4.8 \pm 0.06}$ mean egg infective doses/mouse.
[b] SEM, Standard error of the mean.
[c] $P < 0.005$ versus sham treated.
[d] $P < 0.005$ versus i.p.
[e] $P < 0.01$ versus sham treated.

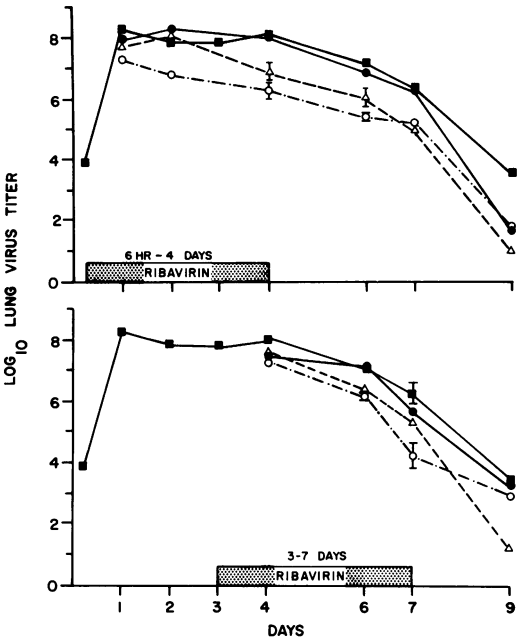

FIG. 1. *Effect of ribavirin on lung virus titers in influenza-infected mice. (Top) Therapy initiated 6 h postexposure to the virus and continued for 4 days. (Bottom) Therapy initiated 3 days postexposure and continued for 4 days. Symbols: sham-treated virus control (■); i.p.-treated (●); aerosol-treated, 80 min/day (△); continuous aerosol-treated (○).*

ters ($P < 0.025$ and $P < 0.005$, respectively) compared with titers from sham-treated virus control mice. The i.p. therapy had no effect on titers of these time periods. When treatment was from 3 through 7 days, only continuously administered aerosols resulted in significantly ($P < 0.05$) lower lung virus titers at 6 and 7 days (Fig. 1, bottom).

Serology. Serum was collected from surviving mice at 42 days after infection to gain insight into the effect ribavirin might have on the immune system. Hemagglutination-inhibition titers of surviving sham-treated virus control mice (Table 2) were equivalent to those of surviving treated mice.

Pathology. By day 3 sham-treated virus control mice had bronchopneumonia with an accompanying bronchial hyperplasia (Fig. 2). The effects of ribavirin treatment initiated 6 h after infection on gross pathology are shown in Fig. 3. Gross lung changes were evident in the sham-treated infected control mice 24 h after histopathological evidence of pneumonia. The lungs of sham-treated control mice were totally consolidated by day 6. Gross lung lesions in mice treated i.p. with ribavirin initially at 6 h were not different from those observed in control mice (Fig. 3). When the compound was administered by aerosol, marked reduction in gross pathology was evident.

Similar reductions in pathology were noted by histological examination at 7, 14, and 21 days. Representative sections of lungs 21 days after infection from each of the four groups of mice initially treated at 6 h are shown in Fig. 4. The sections from the lungs of aerosol-treated mice are essentially normal. Aerosol-treated mice (Fig. 4C and D) did not have bronchial hyperplasia. Bronchial hyperplasia was seen in both the sham-treated control mice (Fig. 4A) and to a lesser extent in i.p.-treated mice (Fig. 4B). Alveolar fetalization was noted in the i.p.-treated groups, but not in aerosol-treated groups. The alveolar fetalization and bronchopneumonia seen at 21 days in the i.p.-treated mice were less than those observed in the sham-treated controls.

DISCUSSION

We have previously reported that rimantadine, amantadine, and ribavirin are therapeutically effective against influenza infection

TABLE 2. *Effect of ribavirin treatment on 42-day-serum hemagglutination-inhibition influenza titers*

Duration of therapy	Route of therapy (geometric mean reciprocal titer)			
	Sham-treated virus control (n = 5)	i.p. treatment (n = 10)	Aerosol 80 min (n = 10)	Aerosol continuous (n = 10)
6 h to 4 days	139 (121,160)[a]	242 (184,320)	160 (138,185)	80 (63,101)
3 to 7 days	127 (101,160)	118 (104,133)	187 (169,207)	139 (109,179)

[a] ± Standard error of geometric mean.

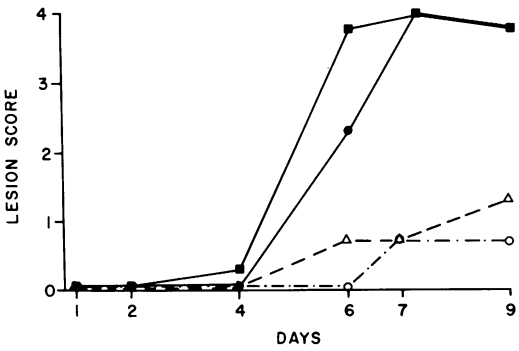

FIG. 3. *Effect of ribavirin on the development of gross lung lesions in influenza-infected mice. Therapy was initiated 6 h postexposure and continued for 4 days. Symbols: sham-treated control (■); i.p.-treated (●); aerosol-treated, 80 min/day (△); continuous aerosol-treated (○).*

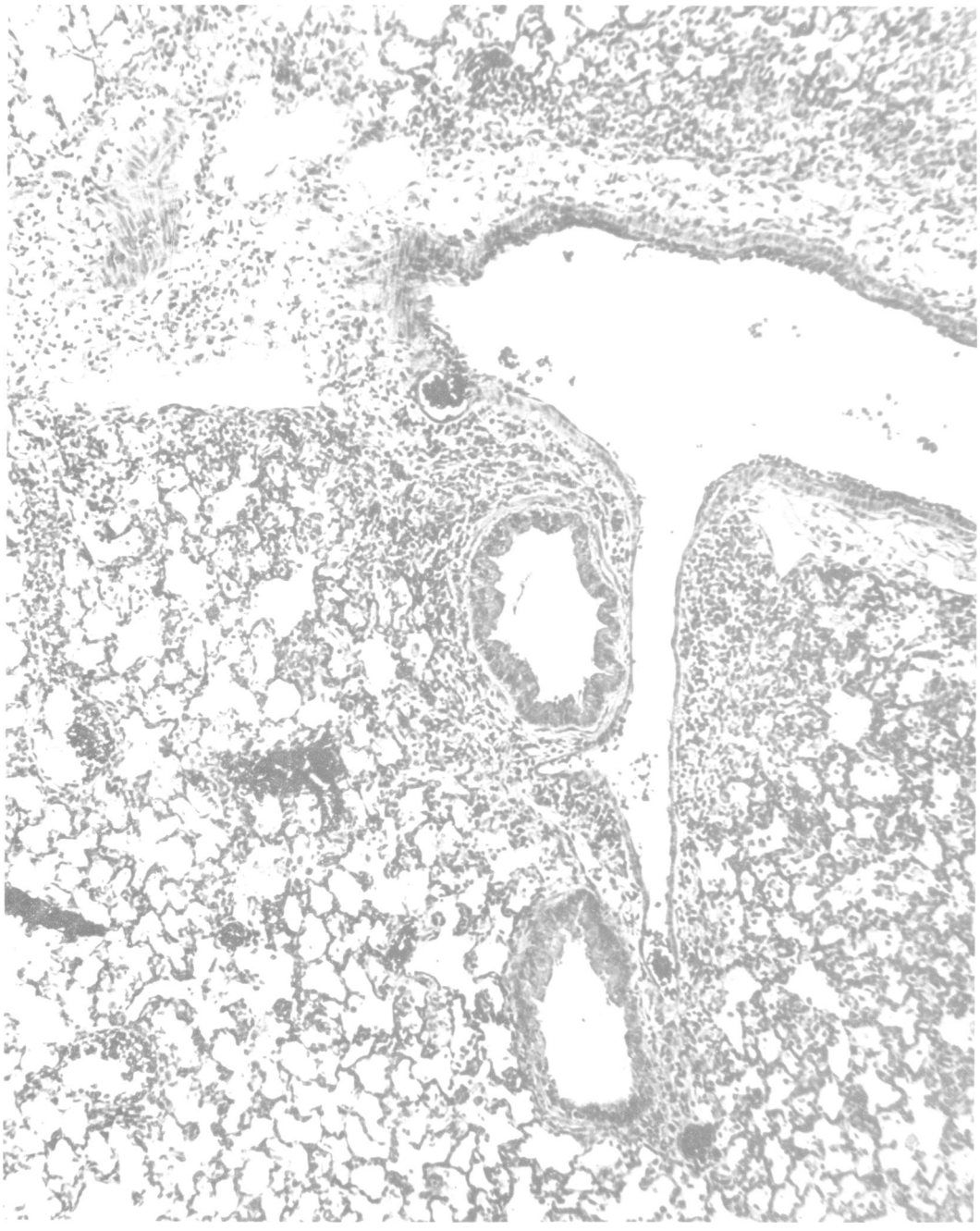

FIG. 2. *Histological changes observed in mouse lungs 3 days postexposure to the virus. Note evidence of bronchopneumonia and bronchial hyperplasia with initial alveolar changes in alveoli closely associated with the bronchi.*

when administered as small-particle aerosols (15, 16). Amantadine was ineffective when given i.p., but resulted in significantly increased survival rates when administered as a small-particle aerosol. Although rimantadine was therapeutically effective both by the aerosol and i.p. routes, it was most effective as continuously administered aerosol for 4 days. Rimantadine did not affect either the concentration of virus or the development of lesions in

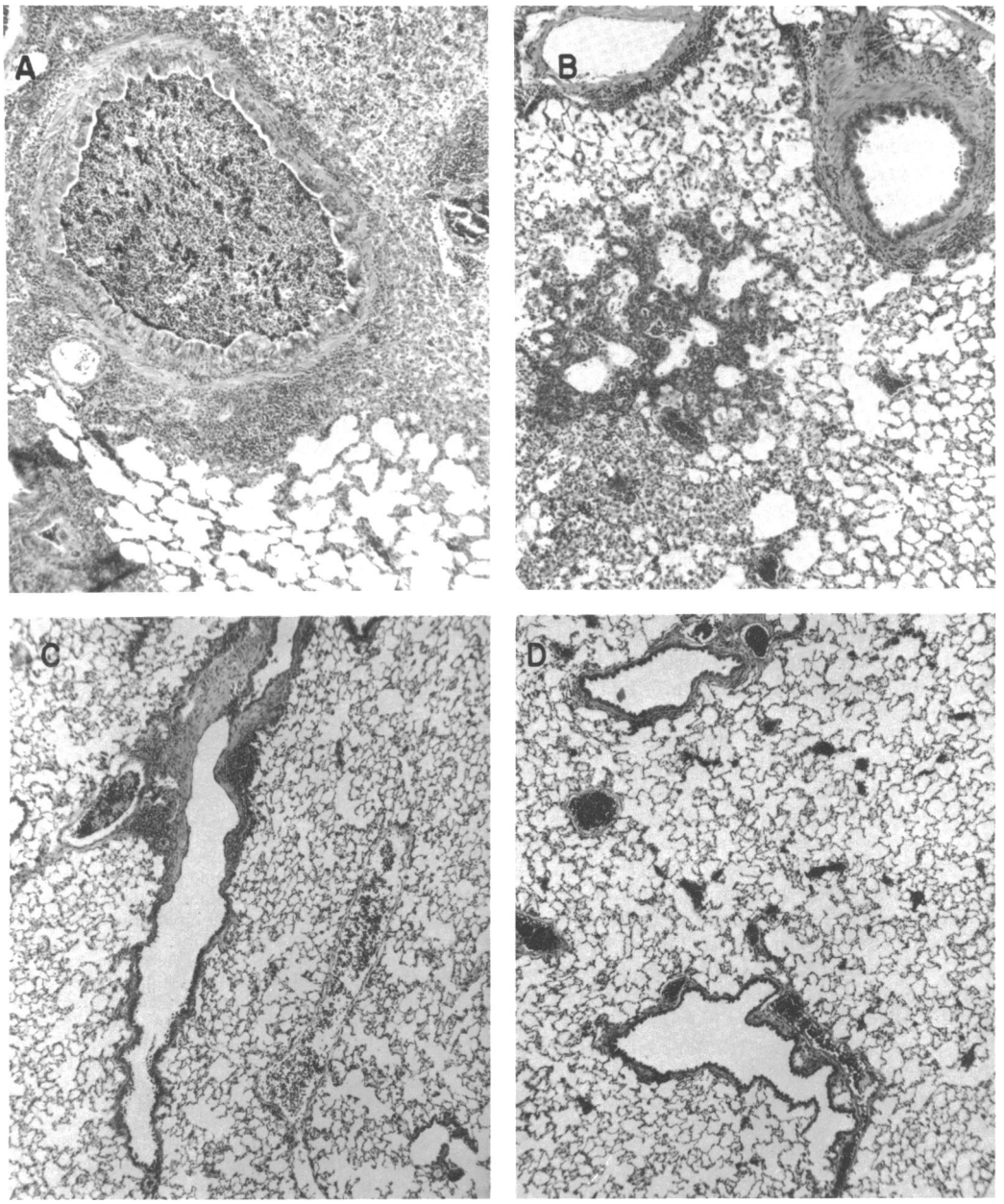

FIG. 4. *Histological differences 21 days postinfection in the lungs of untreated and ribavirin-treated mice when therapy was initiated 6 h postexposure to influenza virus. (A) Lung section from a sham-treated virus control mouse showing severe bronchopneumonia, bronchial hyperplasia, and alveolar fetalization. (B) Section from a mouse treated i.p. showing some bronchopneumonia, alveolar fetalization, and a microabscess. (C) Normal-appearing section from a mouse treated with small-particle aerosols of ribavirin 80 min/day for 4 days. Note normal-appearing bronchial mucosa. (D) Lung section from a mouse treated with continuous aerosols of ribavirin. Note the lack of bronchial hyperplasia and the normal-appearing alveolar duct.*

the lungs regardless of method or schedule of administration. The effect of aerosols of amantadine was similar to that of rimantadine when therapy was initiated at 6 h; however, when therapy was initiated 3 days postinfection, it appeared to reduce the development of lesions and concentration of virus in the lungs (16). In contrast, ribavirin appeared to affect virus titers and the extent of lesions when administered as an aerosol (16).

Our present study has confirmed previous findings with ribavirin and provided detailed information on the extent to which ribavirin affects virus titers and prevents the development of lung lesions when administered at 6 h as an aerosol. After i.p. administration, however, survival was significantly increased, but the development of lesions and virus titers in the lungs were unaffected. Aerosols of ribavirin were most effective when therapy was initiated at 6 h, but therapy initiated at day 3 also significantly increased the percent survival and lowered virus titers. Less extensive development of lung lesions occurred when the drug was administered at 6 h than at 3 days. By day 3 the pathological process was established and, therefore, therapy initiated at 3 days had the effect of expediting resolution rather than preventing lesion development.

We have stated as a hypothesis that the administration of an effective antiviral compound such as ribavirin directly to the surface mucosa of the respiratory tract would be the most efficacious method of administering the drug for the treatment of respiratory infections (16). The present findings confirm this hypothesis and emphasize the beneficial effect of aerosol treatment.

The mode of action of ribavirin is different from that of rimantadine and amantadine. Ribavirin administered as a small-particle aerosol is clearly antiviral in influenza virus-infected mice, whereas rimantadine and amantadine do not consistently lower virus titers in the lungs or affect the pulmonary pathology observed (15, 16). Our observation that ribavirin is effective therapeutically at 3 days, at a time when virus titers have reached a peak in the lungs and pneumonia exists, does not support the suggestion that ribavirin is effective because it prevents virus titers from exceeding critical levels (1). Ribavirin is highly effective after lung virus titers have reached their peak, suggesting that the therapeutic effect of ribavirin involves more than a simple antiviral effect. The dramatic effect of ribavirin in inhibiting developing lung pathology suggests that an "anti-inflammatory" effect may be an important facet of its activity. There is evidence that other drugs effective against influenza may also work in a similar fashion (3).

Ribavirin is an effective antiviral drug for treatment of influenza. It not only increases survival but lowers virus titers and greatly modifies the development of histological lesions in the lungs when administered as a small-particle aerosol. The most efficacious method of administering ribavirin is as a small-particle aerosol (<5 μm), and systems are available to do this (8). Strict attention must be given to the control of particle size of the aerosol and the dosage delivered.

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